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Research Article

Native and invasive raccoon populations host distinct microbiomes that may facilitate invasion

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Abstract

While microbiome associations are known to impact species survival, the extent to which microbiome variation aids in invasion dynamics is largely unknown. Here, we studied the microbiome diversity and composition of native and invasive raccoon populations to assess the microbiome's role in invasion success. We found that native and invasive populations possess highly distinct microbiomes, with the invasive populations displaying greater gut and oral microbiota diversity without signs of instability or functional decline. Significant differences were found between the microbiota of each pair of invasive populations. The core microbiome of introduced populations consisted of a higher number of taxa than that found in native populations, suggesting a lack of microbiome deterioration in invasive populations. However, this may also reflect insufficient time for the establishment of a stable microbiome. Nevertheless, we found no evidence of reduced diversity of the microbiota linked to the population bottleneck or of increased microbiome dispersion indicating lower community stability. Native populations exhibited no microbiome differentiation between distant locations and showed lower, but still reasonably high, alpha diversity, suggesting the stability of the microbiome. Our findings imply that the invasion process changed the microbiota composition while preserving functional capability across different populations. This study highlights the microbiome as a potential factor aiding the adaptation of invasive populations.

Key words: Invasion, invasive species, microbiome, microbiota, *Procyon lotor*, raccoon

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Introduction

Human activities have greatly increased the rates of migration by deliberately or accidentally transporting and introducing large numbers of species to areas beyond natural biogeographic barriers (Perrier et al. 2020). Invasive alien species (IAS), those that become detrimental to recipient ecosystems, are the second most common threat, after habitat loss, associated with species extinctions since AD 1500 (Bellard et al. 2016). Understanding the factors facilitating adaptation of invasive species outside natural ranges is crucial for mitigating their impact. The factors contributing to IAS success include habitat matching (Coulter et al. 2022) or release from native enemies, giving an advantage to IAS over local species (Liu and Stiling 2006). The maintenance of genetic diversity enabling flexibility in adaptation to novel environmental characteristics is also fundamental to the long-term success of invasive species (Kołodziejczyk et al. 2025).

Host–microbiome associations affect host fitness in multiple ways and can be the source of host plasticity (Kolodny and Schulenburg 2020) if that host encounters novel environmental conditions. Indeed, host microbiomes may rapidly increase the host's tolerance to abiotic stress by increasing thermal tolerance (Fontaine et al. 2022), stimulating immune response (Chiu et al. 2017), protecting the host from pathogens (Pickard et al. 2017), or widening dietary niche by enabling metabolism of novel resources (Henry et al. 2021). Given the close relationship between a host and its dependent microbial community, Rosenberg and Zilber-Rosenberg (2018) state that an organism should be viewed as "the holobiont," with the host and its microbiome functioning as a biological entity. Microbes, with larger effective population sizes and shorter generation turnover, may evolve novel functions faster than their hosts (Koonin and Wolf 2012; Ferreiro et al. 2018). Hosts may acquire these adaptive microbes from the local environment, allowing instant adaptation. Nevertheless, the role of the microbiome in wild populations, and even more so in invasive systems, remains largely unexplored (Couch and Epps 2022).

Within a species, microbial communities can vary and have been shown to be influenced by host genotype (Marino et al. 2017), sex (Cong et al. 2016), host infection status (Jani and Briggs 2014), environmental conditions (Wong and Rawls 2012; Aires et al. 2016), and genotype-by-environment interactions (Macke et al. 2017). Variation reflecting specific habitats, including host diet, plays a large role in shaping gut microbial community composition and structure (Eichmiller et al. 2016). Nevertheless, host populations are characterized by a core microbiome that can be defined as a set of microbial taxa specific to a host or environment (Neu et al. 2021). The core taxa are hypothesized to represent ecologically and functionally important microbes. The size and composition of the core microbiome was shown to predict human gut health (Bäckhed et al. 2012) or the responses of organisms to anthropogenic climate change (Hutchins et al. 2019). A large microbiome core should be indicative of high population performance and well-established host–microbiome associations. On the other hand, it has been proposed that the level of dissimilarity in microbiome composition between individuals may indicate a deteriorated host condition. This can be reflected by an increased variance of microbiota (dispersion effects), resulting in higher inter-individual differences due to microbiome instability (Moeller et al. 2013; Lavrinienko et al. 2020).

Invasion processes are predicted to affect the host microbiome (Aires et al. 2016), and the role of the microbiome in invasion processes is increasingly recognized (Romeo et al. 2025). During introduction and range expansion, invasive hosts bring their microbes with them, but a subset of microbial taxa residing in the native host can be lost upon invasion (Kohl 2020; Zepeda-Paulo et al. 2018) in the same way that genetic diversity is stochastically lost when limited numbers of individuals are introduced into a new habitat (Frankham 2005). A shift in microbiome composition between native and invasive ranges has been observed in recent studies (Romeo et al. 2025). Nevertheless, when the microbiome is densely populated and diverse, as in the case of the gut microbiome, strong host–microbiome associations make such a shift less likely (Kohl 2020). The loss of associated microbes, especially those forming the core microbiome, could potentially affect individual fitness. However, invasive species are also exposed to locally available microbiota, creating a changed microbiome over time. In native populations, on the other hand, we might expect less rich but more stable microbial communities, in which novel competitive interactions between microbes

and between microbes and their hosts had sufficiently long time to establish, as reviewed in Ghoul and Mitri (2016).

In contrast to reduced diversity in the invaded range, microbiome diversity may be higher within invasive hosts due to the formation of new relationships with locally available microbes (Himler et al. 2011) or due to a decrease in immune-mediated control of novel microbes (Foster et al. 2017). Higher individual microbiome diversity could potentially increase host performance (Bestion et al. 2017; Stoffel et al. 2020), increasing the chances of invasive species adaptation with the acquisition of beneficial microbes (Martignoni and Kolodny 2024). For example, the phylogenetic diversity of the microbiome of invasive marine herbivorous fishes increased as populations established further from the native range and the local microbiota became increasingly different from the native microbiome (Escalas et al. 2022). The increase was associated with changes in microbiome functions related to the metabolism of short-chain fatty acids and putatively enabled local adaptation (Escalas et al. 2022). Similarly, the microbiome of two invasive hybrid carp species was characterized by high diversity and a high proportion of genes coding for putative enzymes related to their diet (Zhu et al. 2020). During species invasions, an increase in microbiome diversity may help organisms cope with novel environmental conditions or climate changes, potentially from the formation of novel host–microbiome associations. Changes in microbiota may occur due to the loss of specific taxa during introduction, leading to divergent microbiota compositions between native and invasive ranges. Such shifts may not necessarily correlate with a change in the function of the microbiota. The functional redundancy of bacterial taxa (i.e., members of the community with similar functional niches that can substitute for one another) may allow fulfillment of required genomic pathways in both environments (Louca et al. 2018). Although functional redundancy is a known property of the human microbiome (Tian et al. 2020), it has not yet been validated as a factor facilitating species invasions. On the other hand, positive associations between high microbiome diversity and host performance are not unequivocal (Williams et al. 2024), and a number of studies on animal host microbiomes show opposite results (Nelson 2015). For example, high individual microbiome diversity was shown to be associated with poor health in giant pandas (Williams et al. 2016) and chronic laminitis in horses (Steelman et al. 2012). It could therefore be hypothesized that high microbiome diversity of invasive populations may indicate poor performance in the invasive range.

In this study, we analyzed the microbiomes of the raccoon (*Procyon lotor*) from invasive populations in Europe and native populations in North America to test the hologenome hypothesis of invasion. In general, we hypothesized that the gut and oral microbiome of invasive populations would act in concert with the host genome to facilitate invasion success of the species. The raccoon is an omnivorous species that exhibits high plasticity in food choice (Rulison et al. 2012), making it a good model for studying the role of the microbiome. The first successful introduction of raccoons in Europe occurred in Germany in the 1930s with a limited number of individuals (Jernelöv 2017). Unfortunately, information on the invasion source and their microbiome composition is lacking. Genetic analyses of a large set of microsatellite loci and mitochondrial DNA based on whole-country sampling suggest that the current German population of raccoons was established by at least four small-scale, independent introduction events (Fischer et al. 2017) that led to subsequent mixing of genetically divergent invasive populations and

the recreation of relatively high levels of genetic diversity (Biedrzycka et al. 2014). Over time, the invasive range spread eastward, growing into a viable population in western Poland. In contrast, an isolated and stable population of invasive raccoons in the Czech Republic, established in the early 2000s from individuals that escaped from captivity, exhibited lower genetic diversity (Biedrzycka et al. 2014, 2019). In this study, we compare microbiome diversity between invasive European populations of raccoon and two separate populations from the native range (i.e., Florida and Nebraska). These two native populations are demographically stable and occupy similar habitat despite the large geographic distance between them (Jones et al. 1983; Troyer et al. 2014).

To assess the potential role of microbial diversity in the successful spread of raccoons in the invasive range, we characterized the gut and oral microbiome in native and invasive populations. We sought to test three non-mutually exclusive hypotheses concerning microbiome diversity: (i) demographic processes that shaped the genetic diversity of invasive populations will be reflected in microbiome diversity; (ii) the microbiomes of invasive populations will be differentiated from the native ones as a result of microbial taxa loss and gain, but a certain number of core taxa should be maintained, provisioning crucial microbiome functions; (iii) the differential composition of the population microbiome will affect its functional profile, potentially affecting population performance. Overall, this study provides a framework for evaluating how the interaction of the microbiome and host relates to invasion success.

Materials and methods

Sample collection

From each raccoon individual, we collected oral and fecal swabs. We sampled two populations in their native range, near Melbourne, Florida (FL: 25 fecal and 21 oral swab samples), and between Lincoln and Syracuse, Nebraska (NE: 7 fecal and 9 oral swab samples). From the invasive range, samples were collected from a mixed natural and urban habitat surrounding Kostrzyn, Poland (PL: 93 fecal and 73 oral swab samples), in the area between Sonnenberg and Fulda, Germany (GE: 10 fecal and 16 oral swab samples), and around Tovacov and Troubky, Czech Republic (CZ: 35 fecal and 31 oral swab samples) (Fig. 1). Pairs of swabs (fecal and oral) were taken from each individual. Samples from PL were collected between 2021 and 2022, from CZ in 2021 and 2022, from GE in 2022, and from FL and NE in 2023. Samples from Florida were collected from adults transported to the Florida Wildlife Hospital, Inc., during euthanasia or surgical anesthesia directly after a vehicle collision, while samples from Nebraska were collected as a result of local pest control programs. In the invasive range, samples were obtained thanks to game management activity in Poland, Germany, and the Czech Republic. Sex and age class (juvenile or adult) of each individual from PL and CZ were recorded. All samples were collected with sterile, microbiological-grade transport swabs at a depth of 4 cm from the rectum for fecal swabs or inside the mouth at the inner side of the cheek and gums for oral swabs. All fecal and oral swabs were immediately stored in 96% ethanol to minimize contamination from environmental bacteria. The samples were stored at -20 °C prior to DNA extraction.

Figure 1. Geographic locations of studied populations. Invasive populations: GE (50.3684, 9.0234), PL (52.5645, 14.7138), and CZ (49.4263, 17.3033) on the left; native populations: FL (28.2839, -80.7770) and NE (40.6602, -96.1825) on the right side of the map.

DNA extraction and genetic metabarcoding library preparation

DNA was extracted from swabs using the NucleoSpin Tissue XS kit for DNA from cells and tissue (Macherey-Nagel) according to the manufacturer's protocol in the pre-PCR dedicated area. One extraction blank was included per extraction batch. DNA was quantified using a Qubit Fluorometer (ThermoFisher Scientific). For assessment of microbiome diversity, PCR was performed with modified indexed bacterial primers 16SF_ins0 (GTGCCAGCMGCCGCGGTAA), 16SF_ins3 (TGAGTGCCAGCMGCCGCGGTAA), 16SR_ins0 (GGACTACHVGGGTWTCTAATCC), and 16SR_ins3 (TGAGGACTACHVGGGTWTCTAATCC) (Kozich et al. 2013). Indexing enabled the creation of four combinations of primers, which reduced costs. Amplification was performed using HotStart polymerase (Qiagen) under the following conditions: an initial denaturation step of 15 minutes at 95 °C, 32 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute, with a final extension step at 72 °C for 10 minutes. Each sample was amplified twice, and the resulting products were combined. A blank control was included in each amplification reaction for each primer combination to monitor cross-contamination. PCR products were visually verified via gel electrophoresis on a 1.5% agarose gel and purified using MagSi-DNA beads (magtivio). A second PCR was performed using indexed i5 and i7 primers, enabling two-step indexing. For each plate, we used a combination of eight unique forward and twelve unique reverse primers with 8-base indexes. The protocol was performed as follows: a denaturation step of 15 minutes at 95 °C, 12 cycles of annealing at 95 °C for 30 seconds with HotStart polymerase (Qiagen), 50 °C for 20 seconds, and 72 °C for 20 seconds, followed by a final extension step at 72 °C for 5 minutes. Products were purified, verified, pooled, and quantified using a Qubit Fluorometer. Prepared libraries were sent for sequencing to Novogene (UK) Ltd, using the Illumina NovaSeq 6000 platform in paired-end mode.

Bioinformatic processing

The raw reads had a length of 300 bp and were imported into QIIME2 (version 2024.10; Bolyen et al. 2019), where quality control was performed. Primer removal, denoising, and chimera filtering were performed using the DADA2 plugin within QIIME2. Taxonomic assignment of reads was conducted using a Naïve Bayes classifier trained on the SILVA database (version 138.2) with the q2-feature-classifier plugin. All ASVs (amplicon sequence variants) present in less than 2% of all samples were removed from the dataset. Prior to statistical analysis, sequences classified as eukaryotic, unassigned, or unidentified below the kingdom level were filtered out. Before all analyses, ASV read count data were rarefied to the lowest read count observed in the dataset. The rarefaction threshold was set to 8000. For the rarefaction plot, see Suppl. material 2: fig. S1.

Statistical analysis

Within-population homogeneity tests

Individual microbiome structure and diversity can be affected by host sex (Cong et al. 2016), age (Sadoughi et al. 2022), or season (Maurice et al. 2015). Therefore, we first tested whether the opportunistic mode of sample collection might influence population microbiomes. We performed analyses of alpha and beta diversity using samples from our most well-sampled population, PL. First, we ran analyses separately for fecal and oral samples to test for the effect of 1) sampling year (2021, 2022), 2) sampling season (winter, spring, summer, autumn), 3) sex, and 4) age, to calculate alpha diversity measures using the q2-diversity plugin implemented in QIIME2. We calculated two alpha diversity measures: Shannon's diversity index, which accounts for both the abundance and evenness of the taxa present (Shannon 1948), and Faith's phylogenetic diversity, which incorporates phylogenetic relationships between microbial taxa (Faith 1992). The normality of data distribution was assessed with the Shapiro–Wilk test using the dplyr and ggpubr packages in R (version 4.4.1). For normally distributed data, ANOVA was performed to compare alpha diversity metrics. In cases of a significant ANOVA result, post hoc Tukey HSD (honestly significant difference) multiple comparisons of means were performed. For data with distributions significantly different from normality, transformations such as square root or logarithm were applied. In cases where no transformation normalized the distribution, the Kruskal–Wallis test was performed. Second, we calculated beta diversity measures according to the same grouping using the q2-diversity plugin in QIIME2. We applied Jaccard distance (a qualitative measure of presence–absence taxa between communities; Jaccard 1908), Bray–Curtis distance (a non-phylogenetic quantitative measure accounting for taxa abundance; Bray and Curtis 1957), and unweighted (presence/absence) and weighted (by ASV abundance) UniFrac distances (Lozupone et al. 2006). Differentiation was assessed using PERMANOVA (permutational multivariate analysis of variance, 999 permutations) on Bray–Curtis, Jaccard, unweighted, and weighted UniFrac distances, with the aforementioned variables as predictors. There were no significant differences in alpha diversity measures for either fecal or oral microbiomes between subgroups (see Suppl. material 1), and most beta diversity comparisons were non-significant, with only 17 out of 96 being significant (see Suppl. material 1). Most significant

results (10 out of 96) were obtained for beta diversity comparisons between years (2021 vs. 2022), suggesting differences in composition and/or dispersion of population microbiomes likely driven by temporary environmental conditions. As the majority of samples were collected within a single year (except for PL and CZ), and given the large geographic distances between populations, we did not assume concurrent weather conditions. Therefore, we assumed homogeneity of both fecal and oral microbiomes within populations for all downstream analyses. This allowed us to use the most uniform sample size possible, which is crucial for intra-population comparisons of alpha and beta diversity.

Microbiome differentiation between native and invasive populations and their diversity

Given large sample size discrepancies between sampling sites, we tested whether the two populations from the native region were discrete enough to require separation for future analyses or whether they could be combined into a single population representative of the native distribution. We tested whether within-population microbiome diversity measures (alpha diversity) and between-population measures (beta diversity) performed between native populations showed significant differentiation (see within-population homogeneity tests for methods). Indeed, NE and FL populations did not exhibit differentiation for any indices (see Suppl. material 2: tables S1, S2). We therefore combined samples from these two populations into a single native population, USA (32 fecal and 30 oral samples), for subsequent analyses. To further equalize sample sizes, for all downstream analyses, we randomly subsampled the PL population to 35 and 31 samples for fecal and oral samples, respectively.

To test whether within-population microbiome diversity reflects genetic diversity levels of studied populations, we calculated alpha diversity measures (Shannon's diversity index and Faith's phylogenetic diversity) as described above and visualized results in the ggplot package.

To test whether microbiome differentiation between populations reflects genetic differentiation between them, we calculated beta diversity using Jaccard, Bray–Curtis, unweighted, and weighted UniFrac distances. We performed pairwise PERMDISP (tests of homogeneity of multivariate dispersion among groups) in QIIME2. A significant result indicates that groups differ in the level of variation (Bakker 2024). Higher variability in dispersion of beta diversity distances across populations is hypothesized to be an indicator of lower community stability and could reflect the loss of certain microbiome taxa during population establishment in the invasive range. Further, we tested differences in beta diversity distances using PERMANOVA. Differentiation between samples was visualized using PCoA in the ggplot package in R (version 4.4.1).

Microbiome composition and functional profile

To describe the composition of gut and oral microbiomes in native and invasive populations and assess whether it reflects invasion processes in terms of the number of enriched and depleted taxa, we tested for differential abundance of bacterial ASVs between populations and identified taxa contributing most to inter-population microbiome diversity using ANCOM-BC (analysis of compositions of microbiomes with bias correction; Lin and Peddada 2020) in QIIME2. Analy-

ses were performed on three populations (PL, CZ, and USA), identified bacteria at the family or order level, and used a significance threshold of 0.05. The GE population was excluded because small sample sizes and unequal sample sizes between populations can lead to false positives (Lin and Peddada 2020). The core microbiota (i.e., microbial taxa present across multiple samples within a habitat; Neu et al. 2021) were identified using the phylogeny-based algorithm PhyloCore (Ren and Wu 2016) with a prevalence threshold of 0.9. Due to the use of short 16S fragments and limited availability of marker sequences in microbial reference databases, interpretations below the genus level were considered unreliable. The frequency of core microbiome taxa relative to all taxa present in individual samples was then used to calculate core density. We set the threshold at 0.6 (60% of taxa in the individual belonging to the core microbiome). Estimating whether individuals exhibit a dense core versus a sparse core is necessary to evaluate microbiome stability (Björk et al. 2018). The nearest-sequenced taxon index was calculated for each sequence with Picrust2 (Douglas et al. 2020) (for results, see Suppl. material 3). Most results, however, were below 2, the threshold considered limiting for confident interpretation of taxa.

To determine functional profiles of microbiome communities and assess whether potential differences in gut and oral microbiome composition translate to differences in function, we used Tax4Fun2 software (Wemheuer et al. 2020). We applied the reference database where 16S rRNA gene sequences were clustered at 99% similarity (99NR). The minimum threshold of similarity for ASVs was set at 97%. Functional predictions were generated by assigning ASVs to KEGG Orthology pathways (Wemheuer et al. 2020). Data were analyzed and visualized using microeco, seqinr, and file2meco packages following the microeco (v1.10.0) tutorial, and PERMANOVA was performed using the vegan package in R (version 4.4.1) to assess functional microbiome differences between populations. Because of short read length and limited reference sequences, we could not resolve taxa to the species level, which restricted functional conclusions.

Results

We analyzed a total of 112 fecal swabs and 108 oral swabs from four populations: invasive Polish, Czech, and German, and native USA. The mean raw sequencing depth was 110,649 for fecal samples and 137,475 for oral samples. After filtering, the mean number of reads of ASVs per sample was 68,970 (SD = 50,786) for fecal samples and 80,731 (SD = 58,321) for oral samples. The mean percentage of merged reads was 84% for fecal samples and 76% for oral samples. The total number of reads for fecal samples was 12,966,399 and for oral samples 14,047,213.

Microbiome differentiation between native and invasive populations and their diversity

Although estimates of alpha diversity may be underestimated for GE due to smaller sample size compared to other populations (Willis 2019), we decided to include these results, as the GE population represents the raccoon invasion core and primary site of introduction. Nevertheless, alpha diversity measures for this population should be interpreted with caution. There was a significant difference in alpha diversity in the fecal microbiome between populations for both Shannon's

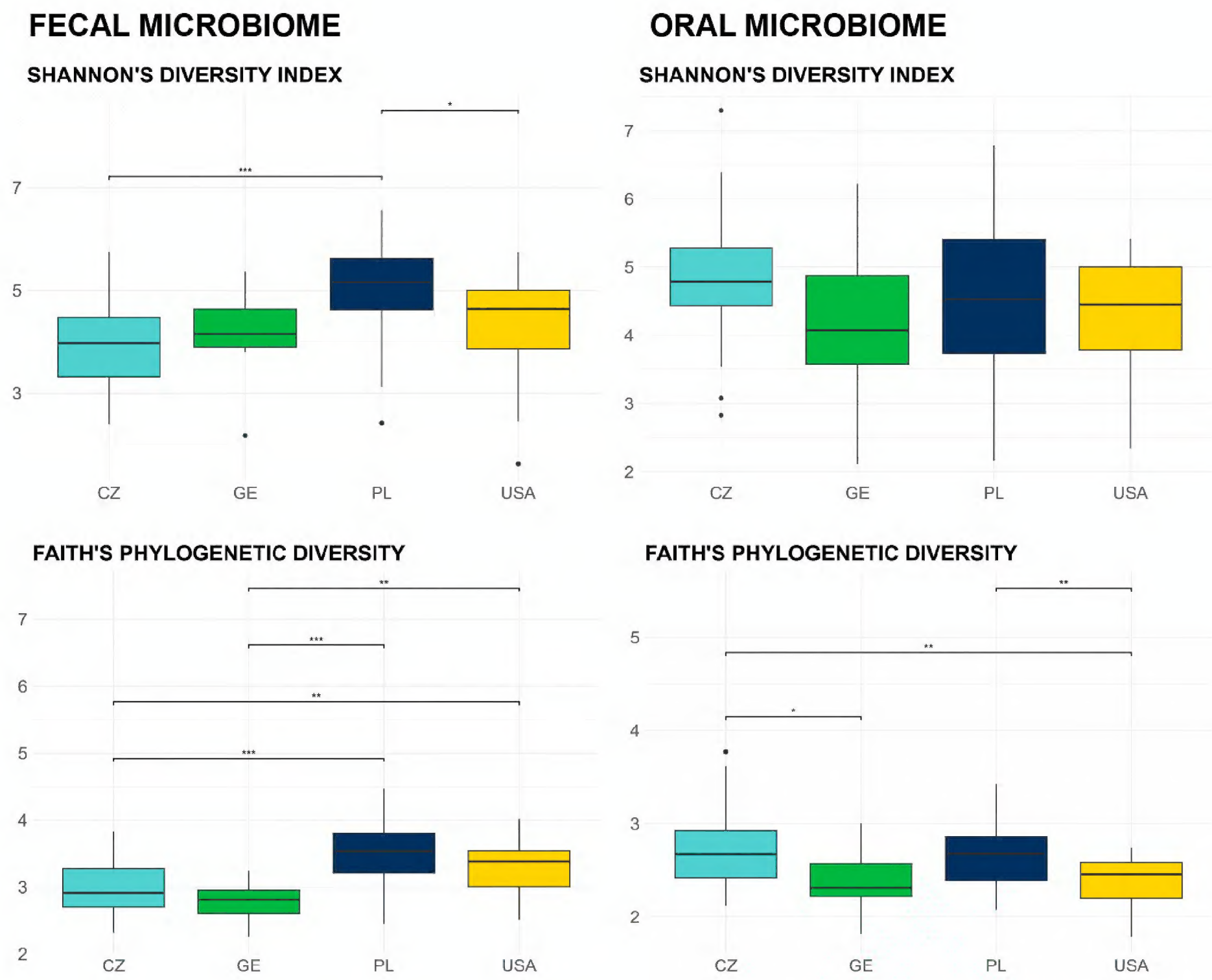

Figure 2. Alpha diversity measures for native and invasive population microbiomes. Measures for fecal microbiomes are displayed in left-side plots, whereas measures for oral microbiomes are displayed in right-side plots. Significant differences were detected between PL–CZ and USA–PL (Shannon's DI for fecal microbiome), between PL–CZ, USA–CZ, PL–GE, and USA–GE (Faith's PD for fecal microbiome), and between GE–CZ, USA–CZ, and USA–PL (Faith's PD for oral microbiome) (Suppl. material 2: table S4).

DI ($F = 6.659$; $p = 0.000368$) and Faith's PD ($F = 11.4$; $p = 1.57^{e-06}$) and in the oral microbiome regarding Faith's PD ($F = 6.984$; $p = 0.000255$) (see Suppl. material 2: table S3). The alpha diversity of the fecal microbiome was significantly higher in PL than in CZ, USA, and GE. However, the alpha diversity of the USA was significantly higher than that of CZ and GE. The alpha diversity of the oral microbiome was significantly higher in CZ than in GE or the USA. However, PL showed significantly higher alpha diversity than the USA (Fig. 2; Suppl. material 2: table S4).

The only significant difference detected by PERMDISP was between the fecal microbiome of GE and USA on unweighted UniFrac distance and between the oral microbiome of CZ–PL and CZ–USA on weighted UniFrac (Suppl. material 2: table S5). PERMANOVA results of diversity measures for these pairs should be approached with caution, as observed differences may be influenced by dispersion rather than shifts in composition. The lack of significant PERMDISP differences for other pairs indicated that differences in composition were not explained by within-group variance. PERMANOVA on unweighted UniFrac detected significant differentiation in both fecal and oral microbiomes between all population

Table 1. Results of beta diversity metrics. Pairwise PERMANOVA results for Jaccard, Bray–Curtis, unweighted, and weighted UniFrac distances on fecal (left) and oral (right) microbiomes. The number of permutations was set to 999; all *p*-values were adjusted with the Benjamini–Hochberg procedure. Asterisks indicate significant results.

			FECAL				ORAL			
	Group 1	**Group 2**	**Sample size**	**F**	**R^2**	**p**	**Sample size**	**F**	**R^2**	**p**
Jaccard distance	CZ	GE	44	1.65	0.0564	0.004*	46	1.906	0.048	0.001*
		PL	67	2.544	0.0447	0.002*	61	2.2	0.036	0.001*
		USA	66	3.325	0.0568	0.002*	59	3.118	0.057	0.001*
	GE	PL	43	1.454	0.0331	0.02*	47	2.129	0.046	0.001*
		USA	42	1.857	0.043	0.004*	45	2.408	0.053	0.001*
	PL	USA	65	2.82	0.0433	0.002*	60	3.136	0.051	0.001*
Bray-Curtis distance	CZ	GE	44	2.444	0.074	0.004*	46	1.981	0.055	0.009*
		PL	67	4.537	0.061	0.002*	61	2.342	0.036	0.001*
		USA	66	5.43	0.084	0.002*	59	2.941	0.051	0.001*
	GE	PL	43	1.298	0.031	0.142	47	2.388	0.046	0.001*
		USA	42	2.159	0.051	0.006*	45	2.53	0.055	0.001*
	PL	USA	65	3.243	0.047	0.002*	60	2.509	0.040	0.002*
Unweighted UniFrac distance	CZ	GE	44	2.484	0.3407	0.004*	46	2.401	0.055	0.002*
		PL	67	4.441	0.581	0.001*	61	2.431	0.054	0.002*
		USA	66	8.081	1.244	0.001*	59	11.194	0.155	0.002*
	GE	PL	43	2.925	0.373	0.001*	47	2.000	0.036	0.002*
		USA	42	4.326	0.683	0.001*	45	5.761	0.120	0.002*
	PL	USA	65	7.161	1.241	0.001*	60	9.245	0.127	0.002*
Weighted UniFrac distance	CZ	GE	44	0.815	0.023	0.544	46	0.822	0.029	0.509
		PL	67	3.327	0.108	0.019*	61	2.685	0.064	0.033*
		USA	66	6.233	0.125	0.003*	59	7.794	0.123	0.003*
	GE	PL	43	1.349	0.058	0.256	47	1.238	0.035	0.313
		USA	42	3.874	0.088	0.019*	45	4.405	0.093	0.01*

pairs, whereas weighted UniFrac distances differed significantly in both fecal and oral microbiomes between CZ–PL, CZ–USA, GE–USA, and PL–USA (Table 1), indicating differences in the most abundant taxa. PERMANOVA results on Jaccard distance were significant in all cases for both fecal and oral microbiomes. Differences in Bray–Curtis distance were significant in all pairwise comparisons for both fecal and oral microbiomes, except for the GE–PL pair in the fecal microbiome (Table 1; Fig. 3). In general, differentiation expressed as unweighted UniFrac distance was higher than that detected by Jaccard distance, indicating that differentiation was driven by more phylogenetically distant taxa (Table 1). Non-significant between-population estimates for Bray–Curtis distances (GE–PL for fecal) and weighted UniFrac distances (GE–PL for fecal; CZ–GE and GE–PL for oral microbiome) indicate that differences were caused by presence/absence of taxa rather than their abundance. For all distance measures, the largest separation was observed between the native and all invasive populations (Table 1).

Microbiome composition and functional profile

The results of the ANCOM-BC differential abundance test (Fig. 4) indicated that more taxa were enriched in PL compared to the USA (in the fecal microbiome, seven taxa; in the oral microbiome, 28 taxa) than depleted (in the fecal microbiome,

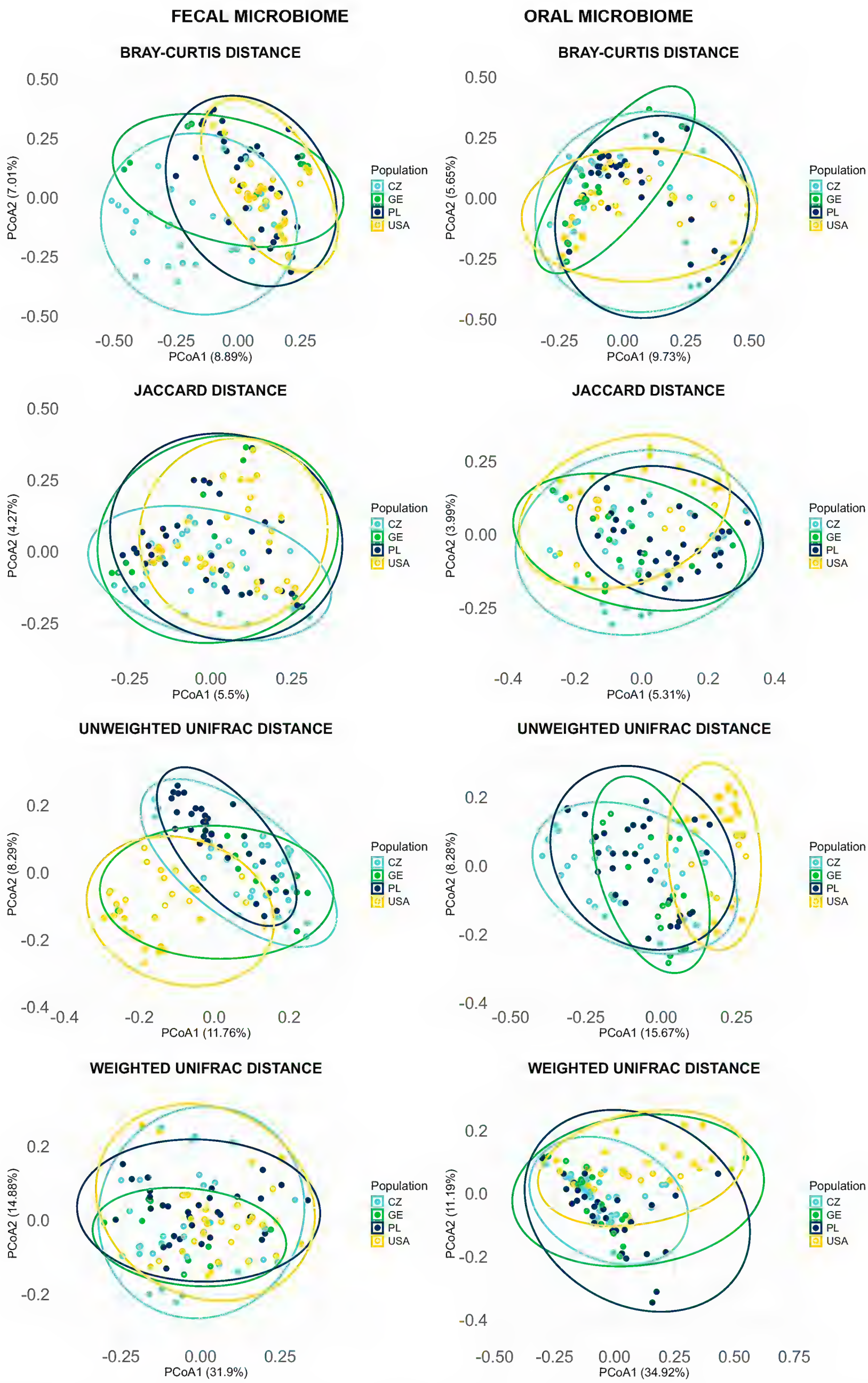

Figure 3. Beta diversity measures for native and invasive population microbiomes. Measures for fecal microbiomes are displayed in left-side plots, whereas measures for oral microbiomes are displayed in right-side plots. The significance of inter-population differences in diversity is given in Table 1.

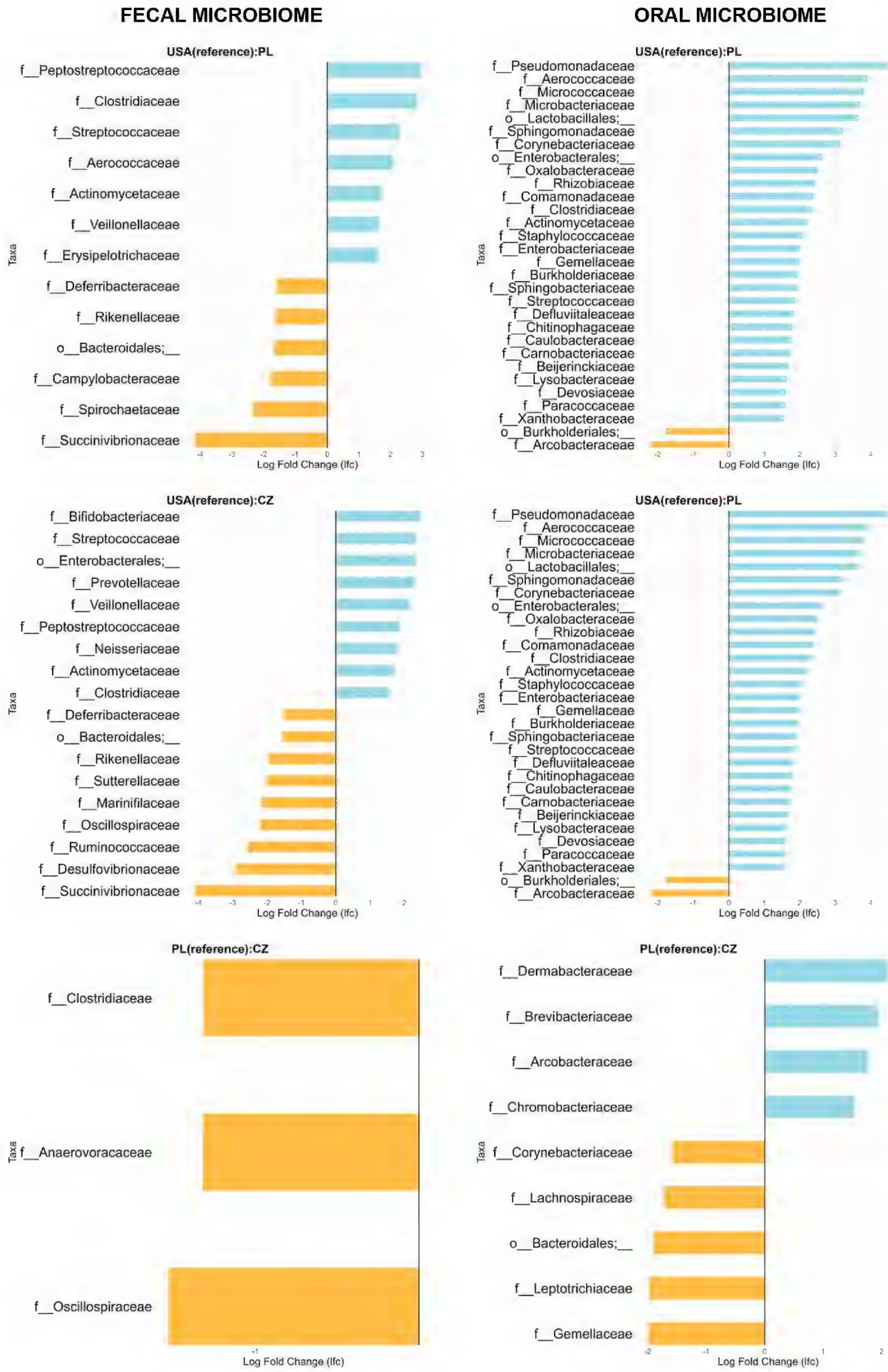

Figure 4. Differential abundance of taxa. Plots display differential abundance of significantly different taxa between populations from fecal (left) and oral (right) microbiomes detected by ANCOM-BC; o – order, f – family. Full taxonomy is shown in Suppl. material 2: tables S6, S7.

six taxa; in the oral microbiome, two taxa). The fecal microbiome exhibited nine enriched taxa and nine depleted taxa when comparing CZ to the USA (Fig. 4). However, comparison of the oral microbiome found that 20 taxa were enriched in CZ and only seven depleted compared to the USA. PL and CZ differed significantly in the abundance of one family, Oscillospiraceae, which was depleted in the CZ fecal microbiome. In the oral microbiome, abundance differed in nine taxa: four were enriched and five depleted in CZ compared to PL (Fig. 4).

In the fecal microbiome, the highest number of core components was detected in GE (22), followed by PL (17) and CZ (14), with the lowest number in USA (13). In the oral microbiome, we detected the highest number of core components in PL (34), followed by CZ (31), GE (17), and, as with fecal samples, the lowest number in USA (16). Six taxa in the fecal microbiome and five taxa in the oral microbiome were shared between all studied populations. Five taxa in the fecal microbiome as well as five taxa in the oral microbiome were present only in the native population. Core taxa overlapping among all invasive populations (excluding native taxa) were represented by four fecal and six oral microbiome taxa (Fig. 5). In the fecal core microbiome of all populations, we detected *Clostridium*, Lachnospiraceae, *Bacteroides*, *Helicobacter*, *Fusobacterium*, and *Escherichia/Shigella*. Taxa assigned to the fecal core microbiome of all invasive populations but absent in native populations were Actinobacteria, *Streptococcus*, Prevotellaceae, and Moraxellaceae. Lactobacillales, Ruminococcaceae, Peptostreptococcales-Tissierellales, *Cetobacterium*, and Sutterellaceae were components only of the native fecal core microbiome. We identified *Streptococcus*, Lachnospiraceae, *Fusobacterium*, *Glaesserella*, and *Moraxella* in the oral core microbiome of all populations. Components of the oral core microbiome of all invasive populations were *Bacteroides*, *Helicobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Escherichia/Shigella*, Lysobacteraceae, and *Pseudomonas*. The native oral core microbiome consisted of Actinobacteria, Mycoplasmataceae, Campylobacterales, Leptotrichiaceae, and Neisseriaceae.

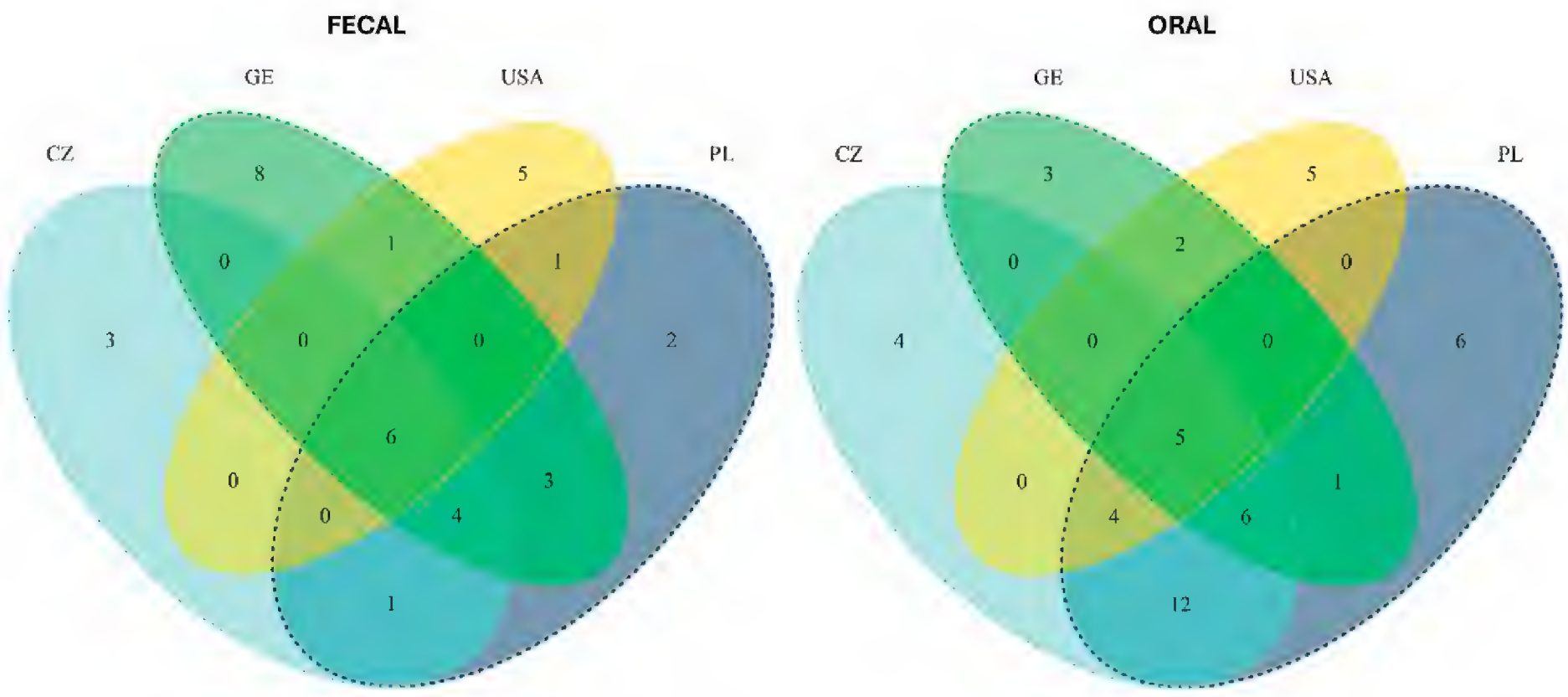

Figure 5. Core microbiota by population in fecal and oral microbiomes, taking into account ecological coherence of higher-rank taxa and calculating core taxa at different taxonomic levels. The number of core microbiota attributed to each studied population is shown for fecal (left) and oral (right) microbiomes. Core microbiome taxa are listed in Suppl. material 2: tables S8, S9. The most common taxa in the fecal core microbiome were *Bacteroides*, *Fusobacterium*, and *Helicobacter*, whereas in the oral core microbiome they were *Glaesserella*, *Moraxella*, and *Porphyromonas*.

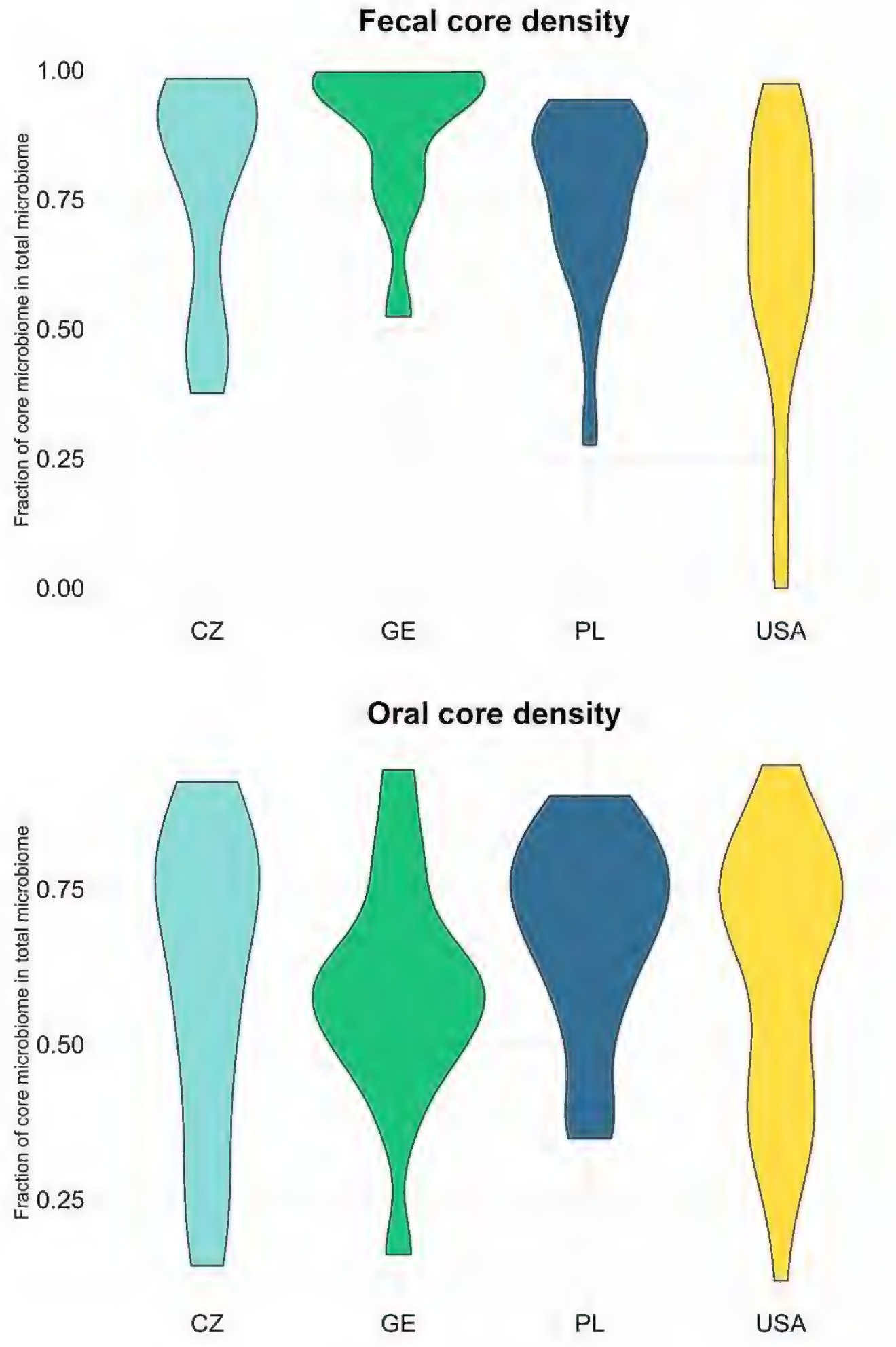

Figure 6. The core density in populations. The density of fecal (top) and oral (bottom) core microbiomes. The frequency of the core microbiome within the total microbiome composition is shown for individuals from four studied populations.

Core density was calculated as the frequency of taxa belonging to the core microbiome present in each individual from that specific population (Fig. 6). In the native population, a core density ≥0.6 was detected in most individuals (20 out of 32 in the fecal microbiome and 18 out of 29 in the oral microbiome). Among invasive populations, the number of individuals with core density ≥0.6 was even higher: in PL, 31 out of 35 individuals in the fecal microbiome and 23 out of 31 in the oral microbiome; in CZ, 26 out of 35 (fecal) and 17 out of 31 (oral); and in GE, 9 out of 10 (fecal) and 6 out of 16 (oral).

The functional microbiome analysis revealed that over 70% of pathways present in each population were related to metabolism (Suppl. material 2: figs S3, S4). At a more detailed level, pathways linked to global and overview maps, carbohydrate metabolism, amino acid metabolism, and membrane transport were most frequently represented (Suppl. material 2: figs S3, S4). PERMANOVA revealed marginally significant differences in pathway predictions between PL–GE, PL–USA, and GE–USA in the fecal microbiome and between CZ–USA and PL–USA in the oral microbiome (Suppl. material 2: table S10).

Discussion

In this study we used raccoons as a model system to investigate microbiome diversity and composition in native and invasive populations. We sampled populations of raccoons from throughout Europe, where raccoons first invaded approximately 90 years ago, and across two populations from the native range. Raccoons are known to use a wide range of food resources (Bartoszewicz et al. 2008; Rulison et al. 2012), invading a variety of habitats, and should putatively rely heavily on microbial services. We found that native and invasive raccoon populations had highly distinct microbiotas. We detected a significant shift in microbiome composition between native and invasive populations, putatively related to different environmental and dietary niches, but possibly also related to loss and gain of microbial taxa during species invasion. In the native range, we identified gut and oral microbiomes that exhibited less diversity than in Europe and little differentiation among populations, despite the large geographic distance between them. This could suggest that native raccoons have developed strong and stable host–microbiome associations related to their diet, relying on human-related food, and putatively contributing to their success in urban habitats. We found that invasive populations were characterized by higher population microbiome diversity, and the core microbiomes in introduced populations consisted of more taxa than those found in native populations. Despite differences in microbiome composition between native and invasive populations and among invasive populations, we did not detect differences at the functional level. Our results indicate that the raccoon microbiome, both in the native and invasive ranges, may facilitate adjustment to local conditions, although through different types of host–microbiome associations. Below we address each of the three hypotheses tested in this study and how our results provide evidence that invaders can form new microbial associations, which may play a role in invasion success, putatively allowing microbially mediated adaptation to invasive habitats.

Microbiome diversity mirrors invasion pathways

We first hypothesized that processes associated with specific demographic events that shaped the genetic diversity of invasive populations would also be reflected in microbiome diversity. Our findings supported this hypothesis in that we did not detect any apparent signs of decreased microbiota alpha diversity in invasive populations that could be attributed to an invasion-related population bottleneck. Although patterns of microbiome diversity resemble those represented by population genetic diversity (Biedrzycka et al. 2014, 2019; Konopiński et al. 2023), this result does not prove a direct relationship between microbial and specific genetic variants.

Rather, our results show the impact of demographic processes that affected both hosts and their microbiomes. Indeed, the highest alpha diversity of the fecal microbiome was detected in the invasive PL population. Recent studies have shown that spatial proximity of hosts can play an important role in creating microbial diversity by mediating exposure to similar microbes and allowing bacterial taxa transfer between individuals (Phillips et al. 2012; Couch et al. 2020). This could explain the high alpha diversity of the dense PL population compared to the two other invasive populations. Despite originating from the invasion core in Germany around the 1980s (Okarma et al. 2012; Biedrzycka et al. 2014) and being located at the invasion front (Biedrzycka et al. 2020), the PL population is characterized by very high densities (Bartoszewicz et al. 2008; Jernelöv 2017), promoting more intensive social interactions between raccoons (Fisher et al. 2021). In contrast, the CZ population, which exhibited significantly lower alpha diversity than PL, was established about 20 years later (Anděra and Gaisler 2012) by individuals that escaped from captivity, with no signs of intensive expansion or exchange of individuals with other raccoon populations (Konopiński et al. 2023). At the same time, this population had lower genetic diversity (Biedrzycka et al. 2014, 2019). Positive associations between genetic and microbiome diversity have been found in other species such as invasive tunicates (Casso et al. 2020; Goddard-Dwyer et al. 2021) and marine sponges (Marino et al. 2017). Our study is the first to suggest such a pattern in an invasive mammal. However, more studies, including direct comparisons of individual microbiome and genetic diversity, are needed to evaluate this association in invasive species.

Second, we hypothesized that the microbiomes of invasive populations would be differentiated from the native ones as a result of microbe taxa loss and gain. Our results not only indicated a shift in bacterial communities in invasive raccoon populations compared to those from the native range but also revealed significant differences between invasive populations. The results for the oral microbiome closely mirrored those obtained for fecal samples. The most pronounced difference was due to the occurrence of different taxa rather than differential abundance. Because alpha diversity is relatively high throughout the invasive range, differentiation between invasive populations is likely due to the acquisition of local microbial taxa picked up during their expansion route. Similar patterns were found in invasive signal crayfish, where environmental influences impacted microbiome diversity (Dragičević et al. 2021). The differences between invasive and native raccoon populations suggest a shift in the most abundant microbial taxa, indicating a potential dietary change. This shift could be explained by raccoons in their native range consuming more urban waste, reflecting proximity to human settlements. In contrast, invasive populations tend to inhabit a broader range of environments, including forests and wetlands, where their diet is likely more natural and less influenced by human activity. Differences in microbiome composition can also be related to differences in host genetic background. Although we cannot directly compare individual genetic and microbial differentiation, the genetic structure of native and invasive populations – where establishment from genetically divergent populations remains visible despite intensive gene flow between invasive populations (Biedrzycka et al. 2014) – is well mirrored by their microbiome differentiation. The greatest differentiation, both from other invasive populations and from the native range, was observed in comparisons with CZ, reflecting its different invasion pathway and isolation. This variation was evident in both genetic (Biedrzycka et al. 2014) and microbiome between-population diversity (Fig. 2).

Structural and functional differences between native and invasive microbiomes

The identification and study of core microbial symbionts are of particular interest due to the ecological relevance and potential functionality of microbial taxa that consistently occur in specific habitats, but also because they can be used to identify the health status of individuals (Shade and Handelsman 2012; Astudillo-García et al. 2017). The size of an individual's core microbiome (dense core or sparse core) is indicative of microbiome stability and resilience (Björk et al. 2018). Here we found that in all invasive populations, fecal core microbiome density was shifted towards higher values compared to the combined native population, suggesting a high level of stability in the invasive range (Fig. 6). Invasive populations were also enriched in more taxa than the native population (Fig. 4). Moreover, this difference was even stronger for the oral microbiome, where the core was generally sparser than in the fecal microbiome but enriched in even more taxa relative to the native population (Fig. 4). This enrichment probably reflects a more diverse diet in the invasive range, as suggested by studies showing invasive raccoons to feed on diverse food resources available at any given moment (Michler 2018), whereas native populations are more dependent on continuously available human-related food (Bozek et al. 2007). A positive relationship between diet diversity and gut microbiome diversity has been demonstrated in highly mobile hyenas (Theis et al. 2012) and fur seals (Grosser et al. 2019). Additionally, a high number of individuals possessing a large and dense core microbiome may reflect redundancy of core taxa. Phylogenetically diverse microbial groups present in invasive raccoon microbiomes suggest microbiome resilience. Interestingly, the GE population, represented by a smaller sample size than other populations, exhibited lower alpha diversity but had similar density (90%) and the most diverse fecal core microbiome (22 taxa). This may be related to its early establishment and core location, with substantial exchange of individuals from surrounding regions (Fischer et al. 2015). A delay in the acquisition of local microbes has been proposed as an explanation for the lag phase of invasive species, where the time needed for population establishment is tied to the formation of more diverse microbiota (Martignoni and Kolodny 2024).

We further hypothesized that a shift in microbiome composition associated with raccoon invasion to Europe would be accompanied by impaired microbiome function. Analysis of gene pathways associated with microbial communities displayed weak but significant differences in functional capabilities between populations. The majority of pathways were related to metabolism, mainly global and overview maps and carbohydrate and amino acid metabolism, further confirming the role of gut microbial taxa in nutrient decomposition (Carthew 2021). The minor differences in gene pathway abundance (Suppl. material 2: figs S3, S4) visualized between populations may reflect raccoon plasticity in adapting to different environmental conditions via microbiome composition. Nevertheless, this relatively low level of differentiation is putatively associated with functional redundancy (Louca et al. 2018) of taxa within unique microbiomes, allowing similar functional capabilities across communities. Microorganisms with overlapping roles may compensate for the loss of a beneficial strain by replacing it in metabolic pathways (Youngblut et al. 2019).

We also did not observe increased microbiota community dispersion in invasive populations, which is hypothesized to indicate lower community stability (Lavrinienko et al. 2020). Various types of environmental stress can increase variance in microbiota

composition (dispersion effects), resulting in higher inter-individual microbiome differences (Wu et al. 2016; Halfvarson et al. 2017). Our analyses showed that although studied populations differed in microbiome composition, dispersion of identified taxa was relatively uniform, indicating that novel environmental conditions in the invasive range do not pose challenges that would be reflected in dispersion levels. The only significant differences – between CZ and the native population, and between CZ and PL – are putatively related to differences in diet composition (McFall-Ngai et al. 2013).

Specific microbial taxa

Finally, we hypothesized that despite stochastic shifts in microbiome composition or differences related to specific habitats, the core microbiome taxa would be shared between all populations (native and invasive). The core taxa should represent groups important to host biology and therefore be maintained by host–microbiome coevolution despite stochastic changes induced by introduction to new habitats (Shade and Handelsman 2012; Neu et al. 2021). Due to the lack of species-level taxonomic assignments, we could not draw conclusions about specific functions of particular taxa.

In the core fecal microbiome, we detected six taxonomic groups shared across all studied populations and five groups shared among all invasive populations. A similar number of taxonomic groups were shared for the oral core microbiome, although the specific taxa differed. While we cannot definitively determine the functions of the common taxa, some level of congruence suggests that specific groups are maintained despite changes associated with the invasion process. The detection of numerous, but not necessarily overlapping, bacterial taxa in both native and invasive raccoon microbiomes further supports the finding that raccoon populations from both ranges were able to adapt to their respective habitats despite invasion-related shifts in microbiome composition and differences in population-level microbiome diversity.

The characteristics of the microbiome of native populations

The lack of microbiome differentiation between two distant native populations was surprising. This result could, to some extent, be explained by the relatively low sample size of the Nebraska population, which could affect both the Shannon diversity index (Shannon 1948) and PERMANOVA results (Anderson 2014). However, we did not detect lower alpha diversity in the NE population. Further, the spatial analysis of individual microbiome diversity from the two native populations did not suggest differentiation in composition (Bray–Curtis distance, Suppl. material 2: fig. S2). Although this result must be treated with caution, the lack of differentiation, coupled with lower alpha diversity than in invasive populations, may suggest a highly stable and resistant microbiome. Homogeneity of gut microbiomes has been demonstrated for geographically distinct human populations, suggesting high stability (Jackson et al. 2018). In our study, relatively lower microbiome diversity in the native range may indicate adjustment to local conditions, even if associated with a drop in some diversity measures. The omnivorous diet of raccoons – dependent on current food availability and typically including mollusks, water-related invertebrates and plants, and human-related waste – is typical in both native and invasive populations (Rulison et al. 2012). Nevertheless, the native populations sampled

here are closely associated with urban habitats, which may shift their diet towards human-related food. It is known that such simplified diets lead to low microbiome diversity and can eliminate inter-population differentiation. For example, studies on American black bears revealed that consumption of human-provisioned foods was associated with reduced gut microbial diversity (Gillman et al. 2022). Similarly, in Tome's spiny rats, anthropogenic disturbances led to decreased alpha diversity and shifted beta diversity in gut microbiomes (Fackelmann et al. 2021). On the other hand, it was suggested that the ability to exploit novel food resources in new habitats – as reflected by higher alpha diversity in the invasive PL population – likely played a vital role in the recent successful expansion of raccoons (Rulison et al. 2012). This may suggest that, in contrast to native populations, invasive raccoon populations rely on greater food diversity. Nevertheless, more studies of native raccoon microbiomes are needed to confirm this assumption.

Conclusion

There is an established link between gut microbiome composition and host fitness (Gould et al. 2018). However, knowledge of how the establishment of a species in a new range affects its microbiome, and whether the microbiome plays an important role in invasion success, is limited (but see Minard et al. 2015; Casso et al. 2020; Bankers et al. 2021; Goddard-Dwyer et al. 2021; Escalas et al. 2022). Here we found that the invasion process changes the composition of microbiota in an extremely successful invader, the raccoon. Raccoons possess a high level of genomic diversity created through mixing of divergent invasive populations, allowing for rapid adaptation to novel environments from standing genetic variation (Biedrzycka et al. 2014; Konopiński et al. 2023). Together with high reproductive potential (Ritke 1990; Asano et al. 2003) and opportunistic use of food resources (Michler 2018), these characteristics make the raccoon an extremely successful invader. We revealed that raccoons also possess highly diverse gut microbiota, with no obvious signs of instability and with functional capacity not strongly deviating from that of native populations. Our results suggest that the microbiome reflects the diverse diet of invasive raccoons and, along with relatively high levels of genetic variation in neutral (Uller and Leimu 2011) and adaptive regions of the genome (Day 2015), represents another factor facilitating the successful performance and spread of this invasive species.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization: AB. Data curation: JK. Formal analysis: JK. Funding acquisition: AB. Investigation: AB, EH, JK. Methodology: AB, JK. Project administration: AB. Resources: AB, EH. Software: JK. Supervision: AB. Visualization: JK. Writing – original draft: JK, AB. Writing – review and editing: EH.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information. All amplicon DNA sequences have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under BioProject accession no. PRJNA1249228.

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Supplementary material 1

Results of alpha and beta diversity measures on samples from PL populations used to test whether the opportunistic mode of sample collection might influence population microbiomes

Authors: Joanna Kołodziejczyk, Eric Hoffman, Aleksandra Biedrzycka

Data type: xlsx

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Link: https://doi.org/10.3897/neobiota.101.157515.suppl1

Supplementary material 2

Results that are not critical to understanding the general message of the article but provide deeper insight

Authors: Joanna Kołodziejczyk, Eric Hoffman, Aleksandra Biedrzycka

Data type: docx

Explanation note: All results are mentioned in the main text with direct citation.

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Supplementary material 3

Results of the nearest-sequenced taxon analysis

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Data type: xlsx

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